Biochem. J. (1986) 240, 753–763 (Printed in Great Britain)

Calmodulin-binding proteins and calmodulin-regulated enzymes in dog pancreas

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Calmodulin was isolated and purified to homogeneity from dog pancreas. Highly purified subcellular fractions were prepared from dog pancreas by zonal sucrose-density ultracentrifugation and assayed for their ability to bind ^{125}I-calmodulin *in vitro*. Proteins contained in these fractions were also examined for binding of ^{125}I-calmodulin after their separation by polyacrylamide-gel electrophoresis in SDS. Calmodulin-binding proteins were detected in all subcellular fractions except the zymogen granule and zymogen-granule membrane fractions. One calmodulin-binding protein (M_r 240000), observed in a washed smooth-microsomal fraction, has properties similar to those of α-fodrin. The postribosomal-supernatant fraction contained three prominent calmodulin-binding proteins, with apparent M_r values of 62000, 50000 and 40000. Calmodulin-binding proteins, prepared from a postmicrosomal-supernatant fraction by Ca^{2+}-dependent affinity chromatography on immobilized calmodulin, exhibited calmodulin-dependent phosphodiesterase, protein phosphatase and protein kinase activities. In the presence of Ca^{2+} and calmodulin, phosphorylation of smooth-muscle myosin light chain and brain synapsin and autophosphorylation of a M_r-50000 protein were observed. Analysis of the protein composition of the preparation by SDS/polyacrylamide-gel electrophoresis revealed a major protein of M_r 50000 which bound ^{125}I-calmodulin. This protein shares characteristics with the calmodulin-dependent multifunctional protein kinase (kinase II) recently observed to have a widespread distribution. The possible role of calmodulin-binding proteins and calmodulin-regulated enzymes in the regulation of exocrine pancreatic protein synthesis and secretion is discussed.

INTRODUCTION

Previous studies in our laboratory have used a system of guinea-pig pancreatic lobules *in vitro* (Scheele & Palade, 1975; Scheele, 1983) to study the events that couple secretagogue stimulation to increased discharge of secretory protein in the exocrine pancreas (Scheele & Haymovits, 1979). These and other studies have demonstrated that stimulation of pancreatic secretion by both cholinergic agents and cholecystokinin-like hormones is mediated by Ca^{2+} recruited from both intracellular (Gardner, 1979; Williams, 1980) and extracellular stores (Kondo & Schultz, 1976; Scheele & Haymovits, 1979). More recently, evidence has been presented that, on stimulation of isolated pancreatic acini with carbamoylcholine, the cytosolic free Ca^{2+} concentration increases simultaneously with amylase release (Ochs *et al.*, 1983). The mechanism by which Ca^{2+} can influence the rate of pancreatic protein secretion is unknown. Among intracellular receptors of Ca^{2+}, calmodulin appears to exhibit the broadest spectrum of activity in regulating enzymes in a Ca^{2+}-dependent manner (Brostrom & Wolff, 1981; Klee *et al.*, 1980). As a prerequisite to postulating a role for calmodulin in the control of stimulus–response coupling by Ca^{2+} in the exocrine pancreas, it is necessary to demonstrate that this tissue contains both calmodulin and proteins that interact with calmodulin in a Ca^{2+}-regulated manner. Therefore we have examined dog pancreas for the presence of both calmodulin and calmodulin-binding proteins, studied the intracellular distribution of calmodulin receptors, and attempted in several instances to identify the biological activities of these receptor proteins. Some of these results have been published in preliminary form (Bartelt & Scheele, 1981).

EXPERIMENTAL

Materials

Bovine serum albumin, DFP, EGTA, Hepes, Triton X-100, lactoperoxidase, DEAE-cellulose and leupeptin were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Acrylamide, bisacrylamide and SDS were from BDH Chemicals, Poole, Dorset, U.K. Standard M_r markers, Affi-gel 15 and protein assay kit were products of Bio-Rad Laboratories, Richmond, CA, U.S.A. Na^{125}I (2Ci/mmol) was obtained from New England Nuclear, Boston, MA, U.S.A. DuPont Cronex Xtra Lite intensifying screens used in autoradiography were the product of E. I. DuPont de Nemours and Co., Wilmington, DE, U.S.A. Fuji-RX X-ray film was a product of Fuji Photo Film Co., Tokyo, Japan. DEAE-Sephadex and CNBr-activated Sepharose were from Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A. Chicken gizzards were obtained from Pel-Freeze, Rogers, AR, U.S.A. Dr. Albert Mannian (National Institutes of Mental Health, Bethesda, MD, U.S.A.) generously supplied us with CAPP. Bovine brain synapsin was generously given by Dr. Mary Kennedy. Partially purified calmodulin-

Abbreviations used: CAPP, 2-chloro-10-(3-aminopropyl)phenothiazine; DFP, di-isopropyl fluorophosphate; DTT, dithiothreitol.

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dependent bovine brain phosphodiesterase was prepared by the method of Klee & Krinks (1978). Myelin basic protein was prepared by the method of Oshiro & Eylar (1970) and phosphorylated with [γ-^{32}P]ATP (Reimann *et al.*, 1971). Myosin light chain was prepared from chicken gizzard by a modification of the method of Grand & Perry (1983).

Isolation of calmodulin from dog pancreas

Calmodulin was isolated from a 100000 g × 1 h supernatant of dog pancreas prepared in 0.25 M-sucrose/20 mM-Hepes (pH 7.5)/1 mM-$MgCl_2$/10 mM-EGTA/25 mM-KCl/1 mM-DFP/1 mM-2-mercaptoethanol by a modification of the procedure of Watterson *et al.* (1976). After the removal by centrifugation of proteins insoluble in 60% -satd. $(NH_4)_2SO_4$, the supernatant was acidified to pH 4.0 to precipitate calmodulin. The precipitate was collected by centrifugation, dissolved in 100 mM-Tris/HCl (pH 8.0)/1 mM-EGTA/1 mM-2-mercaptoethanol/1 mM-DFP, titrated to pH 7.0, and dialysed against 5 mM-Tris/HCl (pH 8.0)/125 mM-KCl/0.5 mM-EGTA/0.5 mM-2-mercaptoethanol/0.1 mM-DFP. The dialysis residue was chromatographed on DEAE-Sephadex equilibrated in 10 mM-Tris/HCl (pH 8.0)/250 mM-KCl/1 mM-EGTA/1 mM-2-mercaptoethanol/0.1 mM-DFP and developed with a linear gradient of 0.25–0.6 M-KCl in this buffer. Calmodulin was eluted at 0.55 M-KCl and detected by activation of calmodulin-depleted bovine brain phosphodiesterase as described by Grab *et al.* (1979), and by electrophoresis of samples of fractions in polyacrylamide gels under non-denaturing conditions (Watterson *et al.*, 1976). The partially purified calmodulin was dialysed against 50 mM-NH_4HCO_3/0.1 mM-DFP and freeze-dried. Calmodulin was purified to homogeneity by Ca^{2+}-dependent affinity chromatography on CAPP–Sepharose (Jamieson & Vanaman, 1979). A column of CAPP–Sepharose prepared by the method of Cuatrecasas & Anfinsen (1971) and containing 50 nmol of CAPP/ml of resin was equilibrated in 10 mM-Tris/HCl (pH 8.0)/1 mM-2-mercaptoethanol/1 mM-$MgCl_2$/2 mM-$CaCl_2$ (buffer F). Partially purified calmodulin was dissolved in buffer F and was applied to a CAPP–Sepharose column. The column was washed with buffer F, followed by buffer F containing 0.2 M-NaCl, until no further u.v.-absorbing material was eluted from the column. The eluent was changed to buffer F containing 2 mM-EGTA in place of 2 mM-$CaCl_2$. Homogeneous dog pancreatic calmodulin was eluted from the column.

Preparation of subcellular fractions from dog pancreas

Fresh pancreatic tissue was minced, suspended in 5 vol. of 0.25 M-sucrose/1.5 mM-$MgSO_4$/1 mM-DTT (medium A) and passed through a tissue press (1.5 mm perforations). The tissue suspension was homogenized with a Teflon/glass tissue grinder driven by a motor at 3000 rev./min. The homogenate was centrifuged at 600 g for 20 min. The resulting pellets were re-extracted with 5 vol. of medium A and centrifuged as above. The supernatants were combined and made 2.5 mM in EDTA. This postnuclear-supernatant fraction was used to prepare the zymogen-granule (ZG), mitochondrial (M) and postribosomal-supernatant (PRS) fractions.

Zymogen granules and zymogen-granule membranes. By a procedure described by Schneider & Smith (1977) and modified by Brockmeyer & Palade (1979), zymogen granules were isolated by zonal density ultracentrifugation. A 1 litre linear gradient of 9–45% (w/w) sucrose containing 5% Ficoll, 1 mM-EDTA and 1 mM-DTT, pH 6.65, was pumped into a Beckman Ti 15 rotor, equipped with a B XXIX liner and core, from the edge by using a Beckman Spinco gradient maker equipped with a template to form a linear gradient adjusted for the shape of the rotor. After introduction of the gradient, the rotor was filled with the 45%-sucrose solution. The sample (150 ml) of postnuclear supernatant was loaded from the centre of the rotor, overlaid with 50 ml of 8.5% (w/w) sucrose in the buffer, and centrifuged at 19000 rev./min for 60 min. Fractions (25 ml) were collected from the edge of the rotor and analysed as described in Fig. 1. Protein content was measured (Bradford, 1976), and the density of fractions was determined by refractive index. Zymogen granules were located by assaying in the presence of Triton X-100 for amylase (Bernfield, 1955) and for trypsin after activation of trypsinogen with enterokinase (Scheele & Palade, 1975).

Fractions with densities of 1.17–1.19 g/ml and containing zymogen granules were combined, diluted 3-fold with water and centrifuged in an SW40 rotor at 39000 rev./min. for 1 h in tubes containing 2 ml cushions of 45% sucrose/5% Ficoll. Granules were collected from the cushion interface and stored at −80 °C. Granule membranes were prepared by lysis of fresh granules in 200 mM-$NaHCO_3$ (Meldolesi *et al.*, 1971)/1 M-sucrose/1 mM-DFP/1 mM-DTT/1 mM-EDTA. The lysate was overlaid with 0.3 M-sucrose containing DFP, DTT and EDTA in the above concentrations and centrifuged at 145000 $g_{max.}$ for 2 h. Floated membranes were collected at the 0.3 M-/1.0 M-sucrose interface, washed by a 10-fold dilution in 0.25 M-NaBr/1 mM-DTT/1 mM-EDTA and centrifuged at 100000 g for 2 h. The pelleted membranes were suspended in 20 mM-Hepes (pH 7.4)/1 mM-DTT/1 mM-DFP/1 mM-$MgSO_4$ (Hepes buffer) and stored at −80 °C after rapid freezing in liquid N_2.

Mitochondria. Fractions of the zonal sucrose density gradient with densities of 1.13 and 1.14 g/ml contained cytochrome oxidase (Cooperstein & Lazarow, 1951), but no measurable trypsinogen, less than 20 units of amylase/mg of protein and less than 30 μg of RNA/mg of protein (Fleck & Munro, 1962; Schmidt & Thannhauser, 1945). These fractions were combined, diluted 1:1 with water and centrifuged at 13000 g for 10 min. Mitochondria were washed in 15 vol. of Hepes buffer, pH 7.8, containing 2 mM-EGTA, centrifuged, and resuspended in Hepes buffer. Alternatively, they were prepared from a postnuclear supernatant by centrifugation at 8000 g for 10 min. Mitochondria at the top of the pellet were recovered by swirling in 0.3 M-sucrose and centrifuged as described above. Contaminating membranes were separated from mitochondria by flotation as follows: the pellet was resuspended in 1.5 M-sucrose, overlaid with 0.3 M-sucrose and centrifuged at 285000 $g_{max.}$ in a SW40 rotor for 1 h. The pelleted mitochondria were washed as described above and suspended in Hepes buffer. Batches were stored at −80 °C after rapid freezing in liquid N_2. Mitochondrial fractions isolated by either method were of comparable purity as judged by electron microscopy.

Postribosomal supernatant. Zonal sucrose-density-gradient fractions with densities between 1.018 and

1.055 g/ml, containing RNA and amylase activity detectable in the absence of detergent (Fig. 1, fractions 56–62), were combined and centrifuged for 12 h at 90000 $g_{max.}$ in a Ti 60 rotor. Samples of supernatant were stored at −80 °C.

Rough and smooth microsomes, microsomal fractions and ribosomes. Preparations of rough and smooth microsomes were prepared from a postmitochondrial-supernatant fraction by the 'sandwich gradient' technique of Scheele *et al.* (1978). Cycloheximide (3 μg/ml) was included to prevent loss of ribosomes from the rough microsomes during tissue homogenization and subcellular fractionation. The smooth-microsomal fraction was washed with 5 vol. of 0.25 M-NaBr/1 mM-DTT/1 mM-$MgCl_2$/1 mM-EGTA/0.1 mM-DFP, collected by centrifugation at 100000 *g* for 1 h and stored suspended in Hepes buffer. Ribosomes were prepared by centrifugation of a postmicrosomal-supernatant fraction containing 0.6 M-sucrose for 2.5 h at 100000 *g*. The ribosomes were washed in 5 vol. of Hepes buffer, pH 6.8, containing 2 mM-EGTA, collected by centrifugation at 100000 *g* for 2.5 h and resuspended in Hepes buffer containing 0.6 M-sucrose. All fractions were stored at −80 °C.

Electron microscopy

Suspension of particulate subcellular fractions were diluted with an equal volume of 0.2 M-sodium cacodylate (pH 7.3)/2% (v/v) glutaraldehyde/2% (v/v) formaldehyde, fixed for 18 h at 4 °C and centrifuged at 285000 $g_{max.}$ for 1 h in an SW40 rotor. Pellets were post-fixed in 1% OsO_4 in 0.1 M-veronal acetate buffer, pH 6.5, for 1 h at 23 °C. Samples were rapidly dehydrated and embedded in Epon. After polymerization, sections were prepared from blocks with a Sorvall MT-2 ultramicrotome, stained with uranyl acetate and lead citrate and examined in a JEOL 100B electron microscope at 60 kV.

Detection of calmodulin binding to proteins separated by SDS/polyacrylamide-gel electrophoresis

Bovine brain calmodulin was isolated as described by Watterson *et al.* (1976) and purified by affinity chromatography on CAPP–Sepharose as described above for dog pancreatic calmodulin. Calmodulin was iodinated with 1 mCi of $Na^{125}I$/mg by using lactoperoxidase and H_2O_2 (Thorell & Johnsson, 1971). Polyacrylamide-gel electrophoresis in the presence of SDS was performed by the method of Maizel (1969). Binding of ^{125}I-calmodulin to proteins separated by SDS/polyacrylamide-gel electrophoresis was performed as described by Carlin *et al.* (1980), with the following modification: bovine serum albumin (1 mg/ml) was included in the buffer before, during and after incubation with ^{125}I-calmodulin, and the length of incubation and washing was increased to 24 h. After drying, gels were subjected to autoradiography at −80 °C by using an intensifying screen.

Binding of ^{125}I-calmodulin to subcellular organelles

Samples of subcellular organelles containing 100 μg of protein were preincubated in Beckman Spinco polycarbonate tubes (9 ml) with 1 ml of 20 mM-Hepes (pH 7.4)/100 mM-KCl/1 mM-$MgCl_2$/0.1 mM-DFP/1 mM-DTT/bovine serum albumin (8 mg/ml) containing either 1 mM-$CaCl_2$ or 1 mM-EGTA, for 15 min at 37 °C with mechanical agitation. The incubations were conducted for 30 min after the addition of 2.5 μg of ^{125}I-calmodulin (150 μM). Tubes were placed on ice, diluted with 1 ml of cold incubation buffer and centrifuged for 20 min at 100000 *g*. Pellets were washed twice by resuspension in 1 ml of incubation buffer, centrifuged at 100000 *g* for 1 h, and radioactivity was measured with a γ-counter. Tubes containing no subcellular fractions were counted for radioactivity, and these values were subtracted from the values obtained. Since the minimal level of detection was 1 ng of calmodulin bound and samples of subcellular fractions routinely contained 100 μg of protein, only values greater than 10 ng bound/mg of protein were considered significant. In competition experiments, 25 μg of unlabelled calmodulin was included in the reaction mixture.

Affi-gel–calmodulin affinity chromatography

A postmicrosomal supernatant was prepared from 5 g of dog pancreas in homogenization medium and was fractionated by $(NH_4)_2SO_4$ precipitation. The 0–55%-satd.-$(NH_4)_2SO_4$ precipitate was resuspended and depleted of calmodulin by chromatography on DEAE-cellulose in 25 mM-Tris/HCl (pH 7.5)/175 mM-NaCl/1 mM-$MgCl_2$/1 mM-EGTA/0.5 mM-DTT/0.2 mM-DFP. The eluate was made 1.5 mM in $CaCl_2$ and applied to a 1 cm × 10 cm column of Affi-gel–calmodulin (2 mg of calmodulin/ml of resin). Calmodulin-binding proteins were eluted in the same buffer containing 2 mM-EGTA. Pooled fractions were assayed for calmodulin-dependent enzyme activities and, after 6-fold concentration by ultrafiltration with an Amicon Centricon device, the protein composition was analysed by SDS/polyacrylamide-gel electrophoresis.

Enzyme assays

Calmodulin-dependent phosphodiesterase (Wolff *et al.*, 1981) and myelin-basic-protein phosphatase (Wolff & Sved, 1985) were assayed as described in the references. Calmodulin-dependent protein kinase activity was assayed at 30 °C for 15 min in a final volume of 50 μl of 25 mM-Pipes (pH 6.8)/10mM-$MgCl_2$/100 μM-[γ-^{32}P]ATP (0.2 μCi/nmol)/0.5 mM-EGTA either in the absence or in the presence of 1.0 mM-$CaCl_2$ and 10 μg of calmodulin/ml. In addition to measuring endogenous phosphorylation of calmodulin-binding proteins, either chicken gizzard myosin light chain (22 μg) or bovine brain synapsin (10 μg) was included as substrate. Incubations were terminated by the addition of 50 μl of 125 mM-Tris/HCl (pH 6.8)/2% SDS/20% (v/v) glycerol/0.002% Bromophenol Blue/100 mM-DTT and heating to 90 °C for 3 min. Samples were subjected to SDS/polyacrylamide-gel electrophoresis. Gels were stained with Coomassie Blue, destained, dried and autoradiographed. Protein bands in the gel corresponding to bands on the autoradiogram were excised from the gel and counted in a liquid-scintillation counter to quantify phosphate incorporation.

RESULTS

Dog pancreas contains calmodulin

The presence of calmodulin was detected in dog pancreas by assaying a postmitochondrial-supernatant fraction for the ability to stimulate, in a Ca^{2+}-dependent

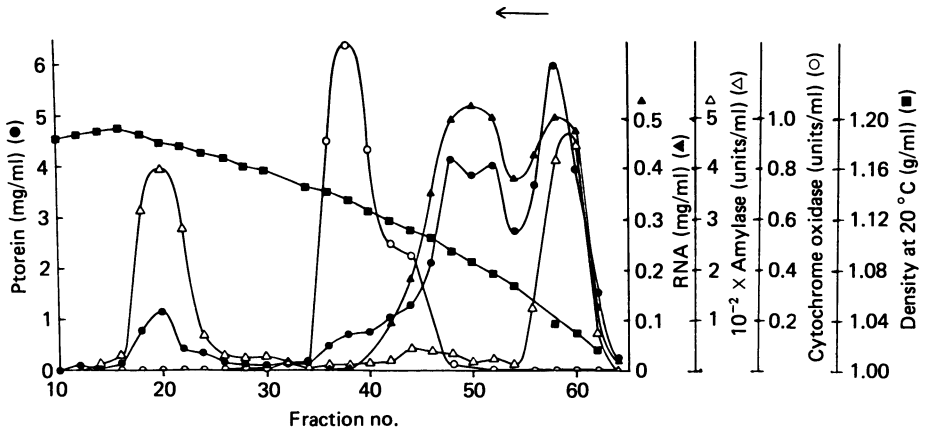

Fig. 1. Separation of dog pancreatic subcellular fractions by zonal sucrose-density-gradient ultracentrifugation

Protein, RNA and enzyme-activity profiles of fractions (25 ml) obtained after centrifugation of a postnuclear supernatant through a linear gradient of 9–45% (w/w) sucrose containing 5% Ficoll are shown. Zymogen granules were collected from fractions 16–24 and mitochondria from fractions 35–39. Fractions 56–62 were used to prepare the postribosomal supernatant. The arrow indicates the direction of centrifugation, from top to bottom.

manner, a partially purified preparation of bovine brain phosphodiesterase. The extent of activation of exogenous phosphodiesterase was determined after correction for endogenous phosphodiesterase activity, and compared with activation by known amounts of purified bovine brain calmodulin. The minimal concentration of calmodulin in dog pancreas, calculated in this manner, was 190mg/kg of tissue, or 11 μM, assuming a tissue density of 1.0 g/ml.

Dog pancreatic calmodulin was purified to apparent homogeneity from a postmicrosomal-supernatant fraction as described in the Experimental section. Some fractions from the DEAE-Sephadex column containing calmodulin also contained S100 proteins (Moore, 1965). Only those fractions devoid of S100 proteins were chromatographed on CAPP–Sepharose, since these proteins are known to bind the phenothiazines in a Ca^{2+}-dependent manner (Levin & Weiss, 1978). Ca^{2+}-dependent binding of calmodulin to CAPP–Sepharose was achieved when the concentration of CAPP was $\leqslant$50 nmol/ml of resin. At CAPP concentrations above 500 nmol/ml, calmodulin could not be eluted with buffer containing EGTA, but required buffer containing 4 M-urea for elution from the column, which resulted in the elution of all proteins bound to the resin non-specifically (results not shown).

Upon electrophoresis under non-denaturing conditions (Watterson *et al.*, 1976), calmodulin from bovine brain and dog pancreas co-migrated, each as a single Coomassie-Blue-stained band. Dog pancreatic calmodulin co-migrated with bovine brain calmodulin and showed the same Ca^{2+}-dependent shift in mobility when samples were subjected to SDS/polyacrylamide-gel electrophoresis. When compared for their ability to activate calmodulin-depleted bovine brain phosphodiesterase, identical quantities of purified dog pancreatic and bovine brain calmodulin stimulated phosphodiesterase to the same extent (results not shown).

Highly purified subcellular fractions are obtained from dog pancreas by zonal ultracentrifugation

Particulate subcellular fractions were prepared as described in the Experimental section. Zymogen-granule and mitochondrial fractions were separated by zonal density-gradient ultracentrifugation. As shown in Fig. 1, this procedure allowed the isolation of zymogen granules free of mitochondria, as judged by the separation of the activities of the marker enzymes amylase and cytochrome oxidase, respectively. Zymogen-granule membranes were isolated from the granule fraction after lysis at alkaline pH. Mitochondria essentially free of zymogen granules and microsomes were obtained by pooling the fractions of the ascending limb of the cytochrome oxidase peak. All fractions were washed with buffer containing EGTA to remove endogenous calmodulin bound in a Ca^{2+}-dependent manner. Electron-microscopic analysis indicated a high degree of cytological purity for each of the subcellular fractions isolated (Fig. 2). The zymogen-granule membranes and smooth microsomes were washed with 0.25 M-NaBr, a chaotropic agent, to remove proteins adsorbed to the surfaces of the membranes.

^{125}I-calmodulin binds to isolated subcellular fractions *in vitro*

In order to determine whether intact subcellular fractions could bind calmodulin, particulate fractions were incubated with ^{125}I-calmodulin in the presence of either Ca^{2+} or EGTA. As shown in Table 1, all of the subcellular fractions except ribosomes and intact zymogen granules were capable of binding measurable amounts of calmodulin in a Ca^{2+}-dependent manner. A significant fraction of total calmodulin binding to both smooth microsomes and mitochondria occurred in the absence of Ca^{2+}. For all fractions tested, binding of ^{125}I-calmodulin was calmodulin-concentration-dependent, saturable and inhibited by the addition of unlabelled calmodulin.

Subcellular fractions contain unique sets of calmodulin-binding proteins

Subcellular fractions of dog pancreas were examined for the presence of calmodulin-binding proteins, by using the gel-binding procedure developed by Carlin *et al.* (1980). The results are shown in Figs. 3 and 4.

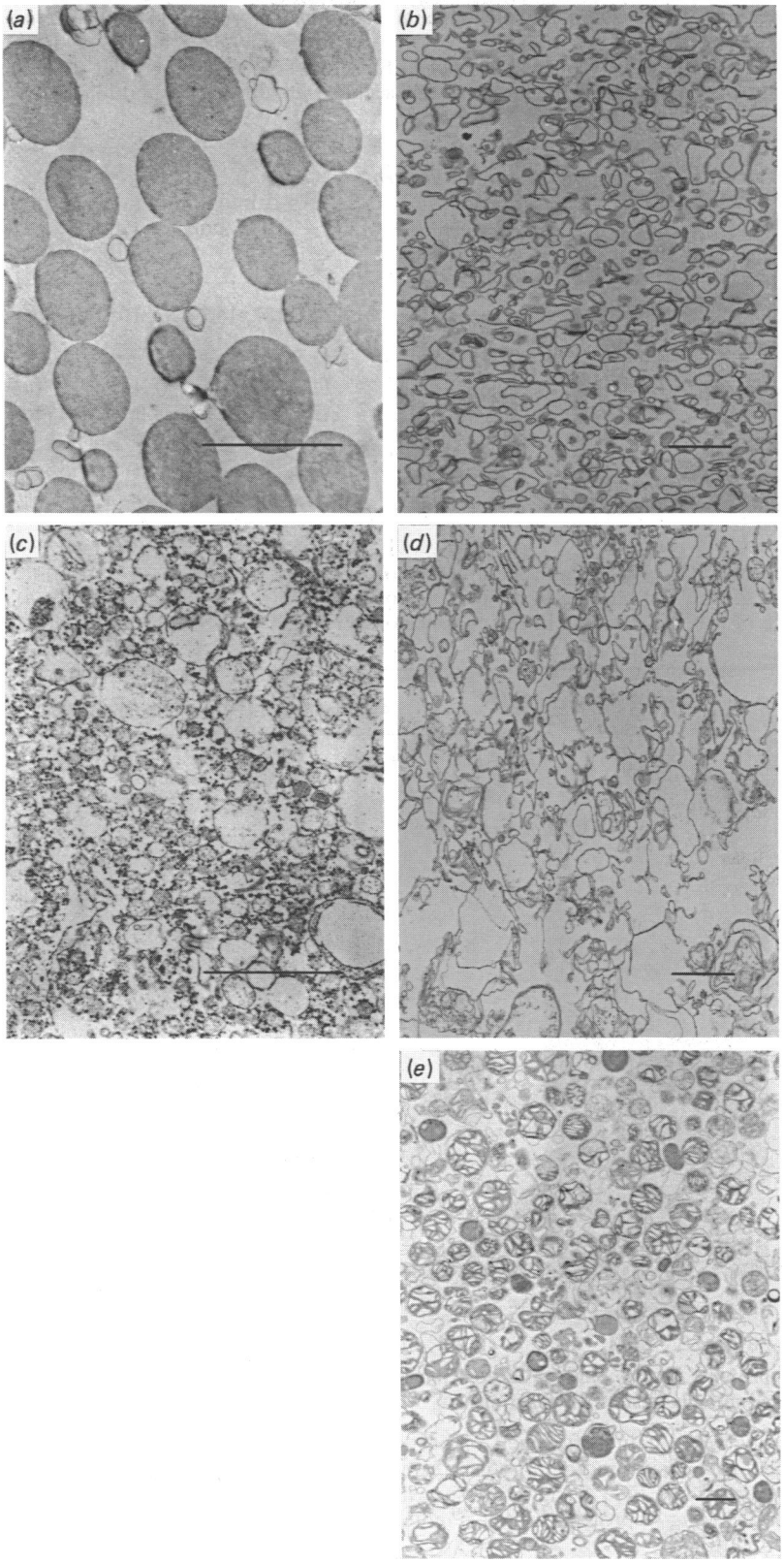

Fig. 2. Cytological purity of dog pancreatic subcellular fractions as examined in the electron microscope

(*a*) Zymogen granules; (*b*) zymogen-granule membranes; (*c*) rough microsomes; (*d*) smooth microsomes; (*e*) mitochondria. Horizontal bars indicate 1 μm.

Table 1. Intracellular distribution of calmodulin binding and calmodulin-binding proteins in dog pancreas

For full details see the text. Fractions were washed in EGTA. Binding of calmodulin greater than 10 ng/mg is above the limit of detection and is considered significant (see the Experimental section). Binding of calmodulin is Ca^{2+}-dependent, except where indicated.

Fraction	^{125}I-calmodulin bound *in vitro* (ng/mg of protein)		^{125}I-calmodulin binding to proteins separated by SDS/polyacrylamide-gel electrophoresis: $10^{-3} \times M_r$
	Ca^{2+}	EGTA	
Postribosomal supernatant	—	—	240, 187, 150, 137, 105, 62, 50, 40.5
Mitochondria	55	48	34, 30*
Smooth microsomes	75	44	240, 187, 162, 150, 137, 125, 105, 88, 62, 50, 43, 34, 30, 23.5, 18.5, 17†
Rough microsomes	39	15	50,* 46.5,* 33,* 29.5,* 28.5,* 26.5,‡ 24,‡ 22,‡ 17†
Ribosomes	9	1	50,* 46.5,* 33,* 29.5,* 28.5,* 26.5,‡ 24,‡ 22,‡ 17†
Zymogen granules	11	6	—
Zymogen-granule membranes	44	5	—

* Binding in the presence of $CaCl_2$ > binding in the presence of EGTA.
† Binding in the presence of $CaCl_2$ < binding in the presence of EGTA.
‡ Binding in the presence of $CaCl_2$ = binding in the presence of EGTA.

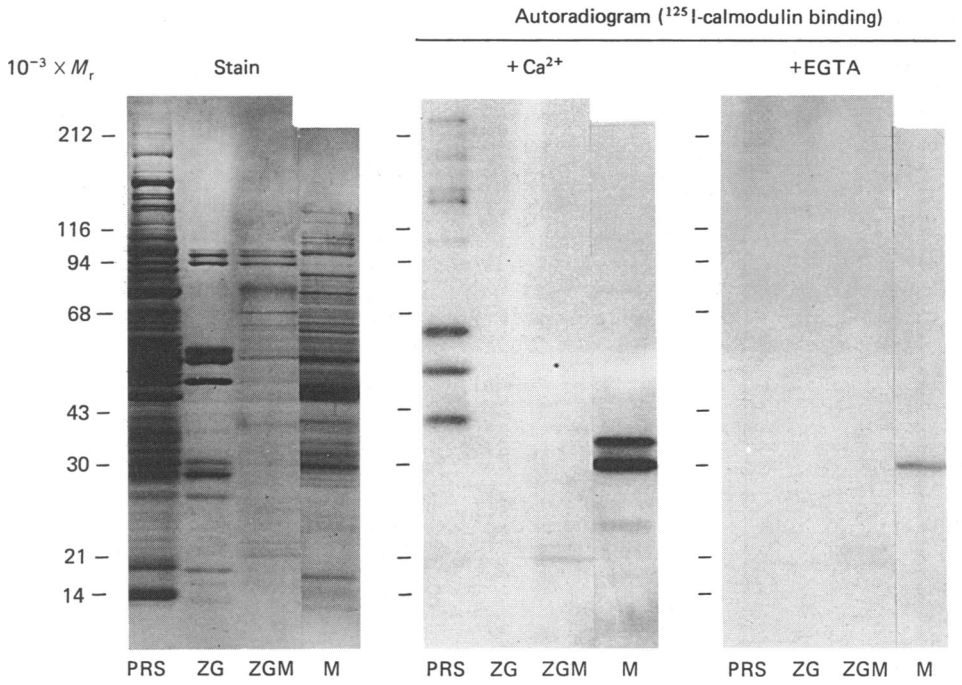

Fig. 3. Calmodulin-binding proteins in dog pancreatic postribosomal supernatant (PRS), zymogen granule (ZG), zymogen-granule membrane (ZGM) and mitochondrial (M) fractions

Coomassie Blue staining pattern (Stain; left panel) of proteins separated by SDS-polyacrylamide-gel electrophoresis on a 5–15%-acrylamide gradient gel and autoradiograms (centre and right panels) of dried gels after incubation with ^{125}I-calmodulin in the presence of $CaCl_2$ (centre) and EGTA (right) are shown. Loading on gel: PRS, 340 μg of protein; ZG, 55 μg of protein; ZGM, 65 μg of protein; M, 130 μg of protein. Lines (–) indicate the positions of protein standards whose M_r values ($\times 10^{-3}$) appear to the left of the Figure.

Postribosomal-supernatant fraction. The gel-binding assay allowed examination of this fraction for calmodulin-binding proteins. In Fig. 3 eight calmodulin-binding proteins are visible in the sample of postribosomal supernatant. They range in M_r from 40500 to 240000, and calmodulin binding to each is Ca^{2+}-dependent. Of these, three proteins, with M_r 62000, 50000 and 40500, are the most prominent.

Zymogen granules, zymogen-granule membranes and mitochondria. As shown in Fig. 3, no Ca^{2+}-regulated calmodulin-binding proteins are readily apparent in

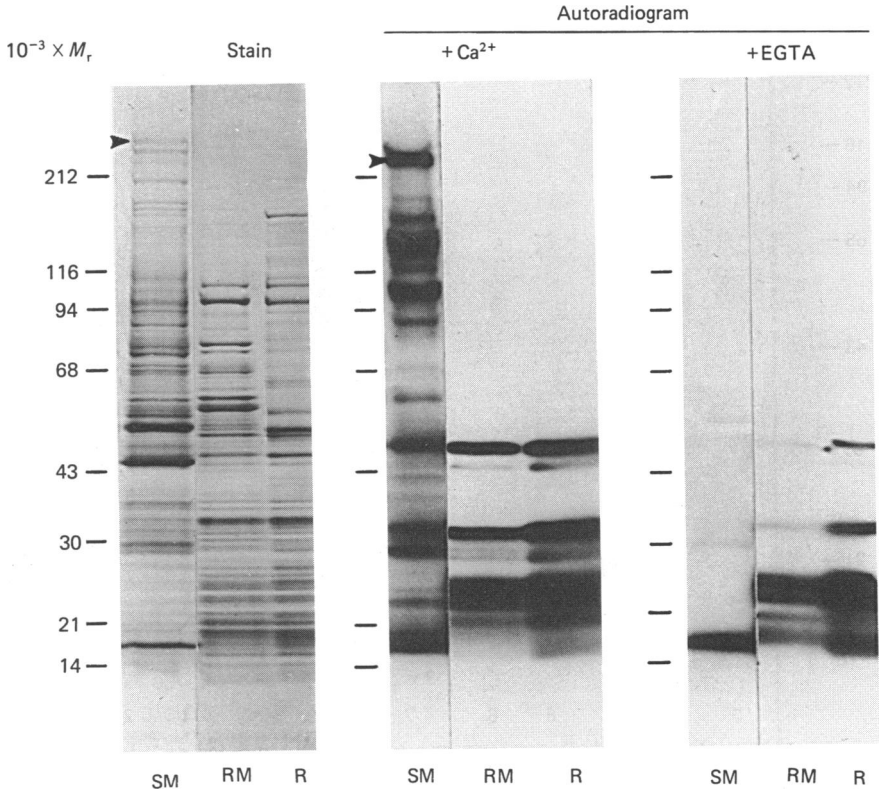

Fig. 4. Comparison of the calmodulin-binding proteins in the 0.25 M-NaBr-washed smooth-microsomal (SM), rough microsomal (RM) and ribosomal (R) fractions of dog pancreas.

Coomassie Blue staining pattern (Stain; left panel) of proteins separated by SDS-polyacrylamide-gel electrophoresis on a 5–15%-acrylamide gradient gel and autoradiograms (centre and right panels) of dried gels after incubation with ^{125}I-calmodulin in the presence of $CaCl_2$ (centre) and EGTA (right) are shown. Loading on gel: SM, 315 μg of protein; RM, 260 μg of protein; R, 250 μg of protein. The stained gel and the autoradiograms have been positioned so that the M_r-17000 proteins observed in the SM fraction appear in a horizontal line. Owing to shrinkage of the dried gel, the M_r-240000 calmodulin-binding protein in the SM fraction seen in the autoradiogram and the corresponding Coomassie-Blue-stained band seen in the left panel (indicated by arrowheads) are not co-linear. Lines (–) indicate the positions of protein standards whose M_r values ($\times 10^{-3}$) appear to the left of the Figure. The autoradiogram of RM and R is a 16 h exposure, and that of SM a 48 h exposure of the same gel.

samples of either the intact zymogen granule or zymogen-granule-membrane fractions when this assay is used. It should be noted, however, that the samples of these fractions applied to the gel contained one-sixth the total protein applied for the postribosomal-supernatant fraction. Two proteins present in the mitochondrial fraction, with M_r 34000 and 30000, bound calmodulin. Binding to the M_r-34000 protein specifically required the presence of Ca^{2+}, and binding to the M_r-30000 protein was considerably greater in Ca^{2+} than in EGTA.

Smooth microsomes. Analysis of the NaBr-washed smooth-microsomal fraction by the gel-binding technique revealed 16 major calmodulin-binding proteins (Fig. 4 and Table 1). At least eight of these proteins co-migrated with calmodulin-binding proteins present in the post-ribosomal supernatant (Fig. 3 and Table 1). Their estimated M_r values are 240000, 187000, 150000, 137000, 105000, 62000, 50000 and 18500. One of the calmodulin-binding proteins, of M_r 17000, bound more calmodulin in the presence of EGTA than in the presence of Ca^{2+}. Binding of ^{125}I-calmodulin to this protein was inhibited by the presence of unlabelled calmodulin (1.5 mM) or trifluoperazine (50 μM) during the radioactive-calmodulin-binding assay. Scanning of autoradiographic data indicated that calmodulin binding to the M_r-17000 receptor was twice as much in the presence of EGTA as in the presence of Ca^{2+}.

Rough microsomes and ribosomes. As shown in Fig 4, the rough-microsomal and ribosomal fractions exhibited approximately the same pattern of calmodulin-binding proteins, indicating that these proteins are ribosomal in origin. The proteins ranged in M_r from 17000 to 50500. Binding of calmodulin to five of these proteins, of M_r 50000, 46500, 33500, 29500 and 28500, was increased in the presence of Ca^{2+} compared with binding in the presence of EGTA. One protein, of M_r 17000, bound more calmodulin in EGTA than in Ca^{2+}. The remaining proteins, of M_r 22000 and 24000–26500, bound calmodulin in a Ca^{2+}-independent manner.

A summary of the estimated M_r values of the calmodulin-binding proteins detected in each of the subcellular fractions after separation in polyacrylamide gels is given in Table 1 along with the Ca^{2+} conditions required for binding.

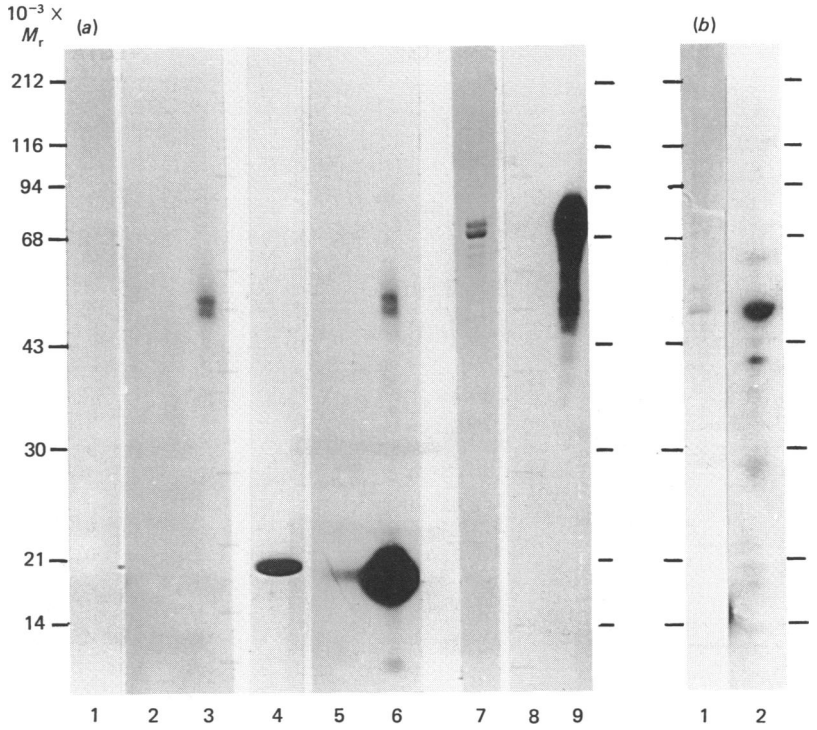

Fig. 5. Characterization of the M_r-50000 calmodulin-binding protein as a calmodulin-regulated multifunctional protein kinase

(*a*) Incorporation of ^{32}P into calmodulin-binding proteins in the absence (lanes 1–3) or in the presence of smooth-muscle myosin light chain (lanes 4–6) or bovine brain synapsin (lanes 7–9). Calmodulin-binding proteins were prepared from a postmicrosomal-supernatant fraction by affinity chromatography on immobilized calmodulin. Phosphorylation reactions were performed as described in the Experimental section in the presence of either 1 mM-EGTA (lanes 2, 5 and 8) or 1 mM-$CaCl_2$ (lanes 3, 6 and 9). Proteins were separated by SDS/polyacrylamide-gel electrophoresis. Lanes 1, 4 and 7, Coomassie-Blue-stained gel. Lanes 2, 3, 4, 6, and 8, 9, autoradiogram of the dried gel. Lower-M_r material in lanes 7 and 9 is degradation products of bovine synapsin. (*b*) Binding of ^{125}I-calmodulin to a sample of calmodulin-binding proteins concentrated 6-fold by ultrafiltration and separated by SDS/polyacrylamide-gel electrophoresis. Lane 1, Coomassie-Blue-stained gel; lane 2, autoradiogram of dried gel after incubation with ^{125}I-calmodulin as described in the Experimental section. Lines (–) indicate the mobilities of protein standards, whose M_r values ($\times 10^{-3}$) are given at the left.

The postribosomal-supernatant fraction contains calmodulin-regulated enzyme activities

Calmodulin-binding proteins contained in a post-ribosomal-supernatant fraction derived from 5 g of pancreatic tissue and prepared by Ca^{2+}-dependent affinity chromatography on immobilized calmodulin were found to contain the following Ca^{2+}- and calmodulin-dependent enzyme activities: myelin-basic-protein phosphatase activity of 450 pmol/min, cyclic GMP phosphodiesterase activity of 750 pmol/min, and protein kinase activity of 2900 pmol/min with gizzard myosin light chain as substrate. In order to characterize the nature of the calmodulin-dependent kinase, proteins isolated by affinity chromatography were assayed for endogenous phosphorylation and the ability to phosphorylate bovine brain synapsin as well as myosin light chain. Results shown in Fig. 5(*a*) indicate that the postribosomal-supernatant contains a protein kinase capable of phosphorylating an endogenous protein doublet with M_r 50000 as well as smooth-muscle myosin light chain and bovine synapsin. The sample of calmodulin-binding proteins was concentrated 6-fold and subjected to SDS/polyacylamide-gel electrophoresis. Prominent among the Coomassie-Blue-stained protein bands were a doublet of M_r 50000. This protein, as well as two others of M_r 61000 and 39000, were prominent among those proteins that bound ^{125}I-calmodulin in a Ca^{2+}-dependent manner when analysed by the gel-overlay technique (Fig. 5*b*).

DISCUSSION

Dog pancreatic calmodulin, when purified to homogeneity, was indistinguishable from bovine brain calmodulin by every criterion tested. This is consistent with the findings of others who have shown that the physical and chemical properties of calmodulins from all sources, from coelenterates to man, are nearly identical (Klee *et al.*, 1980). The amount of calmodulin in dog pancreas (190 mg/kg of tissue) compares well with values determined for rat testis (170 mg/kg; Dedman *et al.*, 1977) and bovine brain (310 mg/kg; Watterson *et al.*, 1976). These tissues have been reported to contain the highest concentrations of calmodulin among mammalian sources (Klee *et al.*, 1980).

Our ability to obtain highly purified subcellular fractions from dog pancreas allowed us to examine the intracellular distribution of calmodulin-binding proteins. Fine-structural and biochemical analyses indicated a high degree of cytological purity for the subcellular organelles

isolated and studied. Calmodulin receptors were detected in each of the subcellular fractions either by binding *in vitro* to isolated organelles or by binding of calmodulin to individual proteins contained in these fractions after their separation by SDS/polyacrylamide-gel electrophoresis. The first method tests the calmodulin-binding capacity of the isolated organelle, whereas the second detects calmodulin binding to specific proteins regardless of their topological orientation in the isolated fraction. The gel-binding assay permits the examination of a population of proteins for the presence of calmodulin-binding proteins. Data collected by this technique are of value in comparing populations of proteins with known purified calmodulin-binding proteins, with a view toward identification of the calmodulin-receptor proteins in that protein population. Except for the similarities observed between the rough-microsomal and ribosomal fractions, each subcellular fraction exhibited a unique profile of calmodulin-binding proteins, suggesting that calmodulin-mediated functions are diverse and vary within the cytological space from one intracellular compartment to another. The two experimental approaches demonstrated the presence of both Ca^{2+}-dependent and Ca^{2+}-independent binding of calmodulin, and allowed us to identify specific binding proteins according to their requirements for Ca^{2+} during the binding process (see Table 1).

The postribosomal-supernatant fraction contained three prominent Ca^{2+}-dependent calmodulin-binding proteins, with apparent M_r values of 40000, 50000 and 62000. Proteins with similar M_r values in a sample of calmodulin-binding proteins prepared by affinity chromatography also bound ^{125}I-calmodulin in a gel-overlay assay. The M_r-50000 calmodulin-binding protein is identified as a calmodulin-dependent multifunctional protein kinase on the basis of: (*a*) calmodulin-dependent autophosphorylation of a M_r-50000 protein doublet present in calmodulin-binding proteins prepared from the PRS by affinity chromatography; (*b*) the presence of calmodulin-stimulated myosin light-chain and synapsin kinase activities; and (*c*) the binding of ^{125}I-calmodulin to the M_r-50000 protein present in the affinity-purified preparation of calmodulin-binding proteins. A M_r-51000 calmodulin-dependent protein kinase, purified from rat pancreas by Gorelick *et al.* (1983), binds ^{125}I-calmodulin in a gel-overlay assay. As with the dog protein, the rat protein was phosphorylated *in vitro* in a Ca^{2+}- and calmodulin-dependent manner. The rat pancreatic kinase has been shown to phosphorylate bovine brain synapsin (Gorelick *et al.*, 1984) and the M_r-29000 ribosomal protein S6 *in vitro* (Gorelick *et al.*, 1983). The latter finding is of particular interest, since carbachol and cholecystokinin stimulation of ^{32}P-labelled pancreatic tissue has been shown to result in increased phosphorylation of ribosomal protein S6 *in situ* (Freedman & Jamieson, 1982). Similar calmodulin-dependent multifunctional protein kinases have been found in both soluble and membrane fractions in rat brain (Kennedy *et al.*, 1983). The brain kinase (also termed protein kinase II) has been shown to consist of both M_r-51000 and -60000 catalytic calmodulin-binding subunits which are autophosphorylated in a calmodulin-dependent manner, whereas the enzyme isolated from rat pancreas appears to exhibit only one subunit (Gorelick *et al.*, 1983). The multifunctional calmodulin-dependent protein kinase from rat brain has been shown to change its mobility during SDS/polyacrylamide-gel electrophoresis, with increasing incorporation of phosphate during autophosphorylation (Kuret & Schulman, 1985). The autophosphorylated M_r-50000 protein doublet observed here could result from multiple phosphorylated forms of the enzyme.

Both calmodulin-dependent phosphodiesterase and protein phosphatase activities were observed among the calmodulin-binding proteins prepared from the postribosomal-supernatant fraction. Calcineurin, a calmodulin-dependent phosphoprotein phosphatase, has been detected in many tissues (Ingebritsen & Cohen, 1983; Tallant & Cheung, 1983), and in brain is found both in the cytosolic fraction and associated with cytoskeletal structures (Wood *et al.*, 1980). It consists of two subunits, a M_r-61000 calmodulin-binding A subunit and a M_r-15000 B subunit (Manalan & Klee, 1983; Stewart *et al.*, 1982). Brain calcineurin A has been shown to bind calmodulin after SDS/polyacrylamide-gel electrophoresis by using the gel-binding assay (Manalan & Klee, 1983). The M_r-62000 calmodulin-binding protein present in both the postribosomal-supernatant and smooth-microsomal fractions, as well as calmodulin-binding proteins prepared from the former by affinity chromatography, may represent the pancreatic counterpart of calcineurin A. Burnham (1985) has isolated a calmodulin-dependent phosphatase from mouse pancreas by affinity chromatography on calmodulin–Affi-gel. The presence of a calmodulin-regulated phosphatase in pancreas is significant in regard to the findings by Burnham & Williams (1982), who have shown that stimulation of isolated pancreatic acini by carbachol, cholecystokinin and the Ca^{2+} ionophore A23187 leads to rapid and reversible dephosphorylation of two cytosolic proteins in parallel with a reversible stimulation of amylase secretion. Calmodulin-dependent phosphodiesterase isolated from bovine brain has been reported to contain two subunits, of M_r 60000 and 63000 (Sharma & Wang, 1985). The M_r-60000 subunit is thought to be the calmodulin-binding moiety. It is possible that the M_r-60000 calmodulin-binding protein observed in the postribosomal supernatant may also be the pancreatic form of calmodulin-dependent cyclic nucleotide phosphodiesterase. The phosphodiesterase and the A subunit of calcineurin isolated from bovine brain were not resolved by SDS/polyacrylamide-gel electrophoresis under the conditions employed here (D. C. Bartelt, unpublished work). Calmodulin-dependent cyclic nucleotide phosphodiesterase has previously been detected in bovine pancreas by Vandermeers *et al.* (1977). This phosphodiesterase may be responsible for the restoration of cyclic GMP to basal concentration after it had been increased with secretagogue stimulation in pancreatic tissue (Haymovits & Scheele, 1976).

The smooth-microsomal fraction in the pancreas represents elements derived from both the Golgi apparatus and the plasma membrane. All but one of the calmodulin-binding proteins detected in this fraction require Ca^{2+} for binding. Two of these protein bands co-migrated with proteins in the postribosomal-supernatant fraction, which could represent calcineurin A and/or calmodulin-dependent phosphodiesterase (M_r 60000) and a calmodulin-dependent protein kinase (M_r 50000). The M_r-240000 calmodulin-binding protein is identified as α-fodrin, on the basis of its co-migration with dog brain α-fodrin during SDS/polyacrylamide-gel

electrophoresis (results not shown) and its ability to bind calmodulin in a Ca^{2+}-dependent manner. Fodrin is the ubiquitious spectrin-like, actin-binding, calmodulin-binding heterodimer associated with the cytoplasmic surface of the plasma membrane (Levine & Willard, 1981; Shimo-Oka *et al.*, 1983; Bartelt *et al.*, 1984). It has also been observed in lower concentrations in soluble fractions (Palfrey *et al.*, 1982). α-Fodrin has been shown to undergo proteolytic breakdown to yield a calmodulin-binding fragment of M_r 150000 (Carlin *et al.*, 1983). It is thus possible that the calmodulin-binding protein of M_r 150000 in the postribosomal-supernatant and smooth-microsomal fractions include fragments of α-fodrin.

One of the calmodulin-binding proteins in the smooth-microsomal fraction bound more calmodulin in the presence of EGTA than in Ca^{2+}. This 17000-M_r Ca^{2+}-regulated trifluoperazine-inhibited calmodulin-binding protein appears by Coomassie Blue staining to be a major protein constituent of the smooth-microsomal fraction. This protein may serve as a calmodulin-binding receptor under resting conditions in the pancreatic acinar cell. A calmodulin-binding protein of M_r 18000 with similar properties has been detected in teleost retina (Nagle & Burnside, 1982). Since the smooth-microsomal fraction is a mixture of Golgi elements and plasma membranes, the exact intracellular localization of the 17000-M_r membrane protein in the pancreas is unknown.

The presence in the postribosomal-supernatant fraction of both calmodulin- and Ca^{2+}-regulated calmodulin-binding proteins is consistent with the hypothesis that calmodulin mediates, in part, stimulus–response coupling in the exocrine pancreas. Significant and unambiguous binding of calmodulin to zymogen granules or proteins associated with zymogen-granule membranes could not be demonstrated in this study. The low yield of zymogen granule membrane precluded the examination of this fraction by the gel-overlay procedure at protein concentrations comparable with those of other subcellular fractions. This may explain the failure to detect calmodulin-binding proteins by this procedure, in spite of significant Ca^{2+}-dependent calmodulin binding to the zymogen-granule membranes *in vitro*. Watkins & White (1985) were able to detect calmodulin-binding proteins in pancreatic-islet secretion granules, but were unable to determine whether the proteins were located in the granule or the granule membrane. Further study is needed to determine if a subset of calmodulin-binding proteins associated with the smooth-microsomal fraction or the postribosomal-supernatant fraction mediate membrane-fusion events (exocytosis) between zymogen granules and the luminal plasma membrane. The observation of calmodulin binding to isolated mitochondria and the presence of two calmodulin-binding proteins in the mitochondrial fraction are of interest with regard to the physiological role of mitochondria in the uptake of Ca^{2+} from the cytosol.

The rough-microsomal and ribosomal fractions showed very similar patterns of calmodulin-binding proteins, suggesting that these proteins are ribosomal in origin. Ca^{2+}-independent calmodulin binding to several of the ribosomal proteins, of M_r 22000–26500, may be due to non-functional electrostatic interactions between calmodulin (pI 4.1) and basic ribosomal proteins (pI $\sim$ 11.0) (Itano *et al.*, 1980; Wool, 1979). However, the binding of calmodulin to five other ribosomal proteins was regulated by Ca^{2+}. These proteins may play a role in the regulation of protein synthesis. Antico-ordinate changes in the synthesis of individual exocrine proteins (increased synthesis of proteinase zymogen forms and decreased synthesis of amylase forms) have been observed during secretagogue stimulation both in guinea-pig pancreatic lobules *in vitro* (Scheele, 1981) and in the rat pancreas *in vivo* (Schick *et al.*, 1984).

We thank Dr. Thomas Brockmeyer for advice and consultation about the zonal-centrifugation procedure for separation of zymogen granules and mitochondria in the pancreas. We thank Dr. Albert Manian for the gift of CAPP and Dr. Mary Kennedy for the sample of bovine brain synapsin. These studies were supported in part by funds from research grant AM 18532 from the National Institutes of Health, U.S. Public Health Service, and a grant from the Cystic Fibrosis Foundation to G.A.S., by grant NS 11252 to D. J. W., and by the Rutgers Medical School General Research Support Fund to D.C.B.

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Received 12 December 1985/16 June 1986; accepted 26 August 1986

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